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Abstract

Purpose To determine the diagnostic accuracy of quantitative real-time PCR assay in diagnosing bacterial vaginosis versus the standard methods: Amsel criteria and Nugent score.

Methods Amsel criteria, Nugent score, and results from the molecular tool were obtained independently from vaginal samples of 163 pregnant women who reported abnormal vaginal symptoms before 20 weeks' gestation. To determine the performance of the molecular tool, we calculated the kappa value, sensitivity, specificity and positive and negative predictive values.

Results Either or both of the Amsel criteria (≥ 3 criteria) and the Nugent score (score ≥ 7) indicated that 25 women (15%) had bacterial vaginosis; the remaining 138 women did not. DNA levels of *Gardnerella vaginalis* or of *Atopobium vaginae* exceeded 10^9 copies/mL or 10^8 copies/mL, respectively, in 34 (21%) of the 163 samples. Complete agreement between both reference methods and high concentrations of *G. vaginalis* and *A. vaginae* was found in 94.5% (154/163 samples, kappa value = 0.81, 95% confidence interval 0.70-0.81). The nine samples with discordant results were categorized as intermediate flora by the Nugent score. The molecular tool predicted bacterial vaginosis with a sensitivity of 100%, a specificity of 93%, a positive predictive value of 73%, and a negative predictive value of 100%.

Conclusions The quantitative real-time PCR assay shows excellent agreement with the results of both reference methods for the diagnosis of bacterial vaginosis.

Keywords: *Atopobium vaginae*; Bacterial vaginosis; Diagnostic accuracy; *Gardnerella vaginalis*; PCR-based test; Pregnancy

Introduction

Bacterial vaginosis (BV) is a common cause of vaginal symptoms in women of reproductive age [1]. Mounting evidence associates BV, determined by clinical Amsel criteria or the Gram stain-based Nugent score, with susceptibility to sexually transmitted diseases, pelvic inflammatory diseases, preterm labor and preterm delivery [2]. Unfortunately, although BV is associated with adverse pregnancy outcomes, regardless of the presence of clinical symptoms, the benefits of screening for and treating BV in pregnant women remain unclear [3-5]. The subjective clinical diagnosis is of limited value in assessing an asymptomatic population, and half the women with BV are asymptomatic [6]. The artificial Nugent score category of intermediate flora has increased the complexity of the clinical approach to vaginal flora: a significant percentage of the women tested have an intermediate score, but this category remains remarkably uncharacterized [7,8,9]. Consequently obstetricians and gynaecologists continue to seek a reliable method for an objective analysis of abnormal vaginal flora.

Recent advances in molecular techniques have increased our knowledge of the microbial ecosystem of BV and associated some bacteria with BV for the first time [10,11]. Of these, one of the most interesting may be *Atopobium vaginae,* an anaerobic and fastidious bacteria recently reported to be highly associated with BV [10,12,13]. Specific proportions of different *Lactobacillus* species are related to the overgrowth of particular BV-associated bacteria, with the disappearance of *Lactobacillus* species related to the development of *Gardnerella vaginalis* and *A. vaginae*, in particular [12,13]. Although *G. vaginalis* and *A. vaginae* are both commonly present in normal flora, high vaginal concentrations of both are highly specific for BV [12,13]. We previously proposed a reliable and accurate molecular tool based on the combination of high vaginal quantification of *G. vaginalis* (DNA level $\geq 10^9$ copies/mL) and *A. vaginae* (DNA level $\geq 10^8$ copies/mL). It resulted in sensitive and specific BV diagnosis when the Gram stain-based Nugent score was used as the gold standard [14]. One of the

limitations of that study, however, was it did not apply a clinical approach to vaginal flora, by using the Amsel criteria, an alternative and commonly accepted diagnostic method for BV, as previous molecular studies have done [11,13,15].

The aim of the present study was to compare the diagnostic accuracy of the quantitative real-time PCR assay for *G. vaginalis* and *A. vaginae* for the diagnosis of BV with the two most commonly accepted methods, the Amsel criteria and the Nugent score.

Materials and Methods

A prospective study of pregnant women took place at the North University Hospital of Marseille (France). Pregnant women were enrolled from September 2008 to November 2009. Vaginal samples were used to screen for BV in women who reported abnormal vaginal symptoms before 20 weeks' gestation. We selected only women with singleton pregnancies and for whom vulvovaginal candidiasis, trichomoniasis and gonococcal infection were ruled out after clinical examination and laboratory tests. The University's institutional review board (Ethics Committee of the University of Aix-Marseille, France) approved the study (in a decision dated September 19, 2008), and all participants provided written informed consent.

Before vaginal sampling, the physician performed a speculum examination to note the appearance of the vaginal discharge, the spontaneous vaginal odour, and the odour after the addition of 10% potassium hydroxide. Vaginal pH was measured with pH strips and a colourimetric scale. After the clinical examination, the physicians took two vaginal samples from each woman at the same time, on two different sterile cytobrushes rotated against the vaginal wall (Scrinet, 5.5 mm, Laboratory CCD International, Paris, France). One sample from the first cytobrush was rolled onto a glass slide for Gram staining with an automated stainer (Model 7320 Aerospray Gram Slide Stainer; Wescor, Logan, UT) and Nugent scoring. The second cytobrush was transferred to a sterile tube containing 500 µl of BME Baral Medium (Invitrogen, Carlsbad, CA) and stored at –80°C until DNA extraction and molecular quantification.

The two methods used to diagnose BV were (1) clinical diagnosis, based on the combination of any three of the following four criteria [16]: vaginal pH greater than 4.5, thin homogeneous

vaginal discharge, clue cells on microscopic examination of vaginal fluid, and "fishy" amine odour after the addition of 10% potassium hydroxide (positive “whiff” test); (2) microbiological diagnosis, based on Gram staining graded according to the Nugent score [17] to determine the presence of normal (score of 0 to 3) or intermediate flora (score of 4 to 6), or BV (score of 7 to 10). Clinicians and laboratory staff made their diagnoses independently, unaware of each other’s results.

The quantitative molecular tool was based on a specific quantitative real-time polymerase chain reaction (qPCR) assay and serial dilutions of a plasmid suspension, as previously described [14]. It targeted *Lactobacillus* species, *A. vaginae*, *G. vaginalis,* and a human albumin gene. **Human albumin gene quantification was used as an internal control to provide evidence for DNA presence and DNA quality.** Briefly, after DNA extraction, a qPCR assay was performed with a Stratagene MX 3000P (Agilent, La Jolla, CA). The amplification program was run at 50°C for 2 min and at 95°C for 15 min, followed by 45 cycles at 95°C for 30 s and at 60°C for 1 min. Five µL of (1) a pure undiluted DNA sample, (2) a DNA sample diluted to 1/10 µL, (3) a DNA sample diluted to 1/100 µL, or (4) the serially diluted plasmid suspension was added to the 20-µL PCR mix that contained the Quantitect Probe PCR Kit mix (Qiagen, Courtaboeuf, France), the two pairs of primers, the two probes, and 100 U Uracil DNA glycosylase (Sigma-Aldrich, Saint Quentin Fallavier, France). All plasmid scale solutions were tested in duplicate. Negative controls were introduced in each reaction plate. To validate the quality and reproducibility of each PCR result, we verified for each reaction plate of each sample that (1) the standard curve remained linear and reproducible, (2) the cycle threshold values for all microorganisms tested in undiluted and diluted samples were reproducible and linear, and (3) the range of values for the number of albumin copies in the vaginal samples used as an internal control was narrow. The

quality and reproducibility of each PCR result were validated, and none of the 163 samples used in this study had to be excluded. The quantitative molecular tool made it possible to reduce the detection limit for all microorganisms to 10 copies per 5 µL (10^3 copies per mL). The final results were expressed as copies of microorganism DNA per mL of vaginal suspension.

To determine the accuracy of the molecular tool for the diagnosis of BV, the subjects were subsequently classified into two groups according to each method for diagnosing BV described above (Amsel criteria and Nugent score): those with BV and those without BV. Agreement between the two reference methods and high vaginal concentrations of *A. vaginae* (DNA level ≥ 10^8 copies/mL) and *G. vaginalis* (DNA level ≥ 10^9 copies/mL) was calculated with the kappa value, determined by the javastat online statistics package [18]. A kappa of 1.0 indicates perfect agreement. Values greater than 0.75 represent excellent agreement and values 0.40-0.75 fair to good agreement [19]. The sensitivity, specificity, and predictive values of *A. vaginae* DNA ≥ 10^8 copies/mL and *G. vaginalis* DNA ≥ 10^9 copies/mL to predict BV were compared with reference methods. The statistical analysis used the UBC Bayesian Calculator Type 2 [20].

Results

The study included all 163 pregnant women. The mean age of the study population was 29 (±7) years. All women included had speculum examinations during which vaginal samples were taken. The Amsel criteria, Nugent score and molecular quantification of *Lactobacillus* species, *G. vaginalis*, *A. vaginae*, and human albumin gene were successfully obtained for each woman.

Table 1 reports the BV results according to both reference methods. Fifteen women had BV according to both the Amsel criteria and Nugent score, while 10 had discordant results according to these two methods: (i) 5 women had normal flora according to Amsel criteria but a Nugent score ≥ 7; (ii) 5 women had BV according to Amsel criteria but a Nugent score < 7. In all, 25 (15%) of the 163 women had BV according to at least one of these methods. The remaining 138 women (85%) did not have BV, as shown by both methods.

The molecular characteristics of the vaginal flora are reported in Tables 2 and 3. No *Lactobacillus* species was detected by the qPCR method in 32 (19.60%) of the 163 samples. The DNA level of *G. vaginalis* equalled or exceeded 10^9 copies/mL for 22 women (13.5%), and that of *A. vaginae* equalled or exceeded 10^8 copies/mL for 30 (18.40%). In all, 34 (21%) of the 163 samples had high vaginal concentrations of *G. vaginalis* or *A. vaginae* or both. The molecular profile of the 25 samples of vaginal flora identified as BV by either the Amsel criteria or Nugent score is presented in Table 3: 18 had no *Lactobacillus* species detected by qPCR, and all had high vaginal concentrations of *G. vaginalis* or *A. vaginae* or both.

Complete agreement between both reference methods for diagnosing BV and the molecular tool (that is, high vaginal concentrations of *G. vaginalis* or *A. vaginae*) occurred in 94.5% (154/163 samples). There was disagreement for nine samples (5.5%). All nine had high vaginal concentrations of *G. vaginalis* (≥ 10^9 copies/mL) or *A. vaginae* (≥ 10^8 copies/mL), did not have BV according to the Amsel criteria, and were categorized as intermediate flora by the Nugent score (Table 2). There was substantial agreement (kappa=0.81; 95% confidence interval 0.70-0.81) between the reference methods and high vaginal concentrations of both microorganisms. Compared with the reference methods, the molecular tool had a sensitivity

of 100%, a specificity of 93%, a positive predictive value of 73% and a negative predictive value of 100% for predicting BV (Table 4).

Discussion

We examined the accuracy of a quantitative real-time PCR assay for *G. vaginalis* and *A. vaginae* for the diagnosis of BV compared with both the Amsel criteria and the Nugent score, both reference methods for this diagnosis. The originality of the present study is its simultaneous consideration of both the clinical characteristics of vaginal flora, according to the Amsel criteria, and the categorization of vaginal flora, according to the Gram stain-based Nugent score, to determine the accuracy of the molecular tool for diagnosing BV.

Good agreement (kappa=0.81) and high sensitivity (100%) and specificity (93%) were reported for the molecular tool in relation to both reference methods. These findings demonstrate the interest of molecular prediction of BV. The advantage of our PCR-based test is its ability to predict BV accurately when the standard methods result in false negatives. In this study, of the 25 flora samples with BV, 10 (40%) had discordant results for the Amsel criteria and the Nugent score (Table 3). Five samples categorized as BV by the Nugent score were normal according to the Amsel criteria. Those results are not surprising because the sensitivity of Amsel criteria for BV diagnosis is reported to be poor [21]. For the Nugent score, 5 samples categorized as BV by the Amsel criteria were rated intermediate according to the Nugent score. The Nugent scoring system is excellent in diagnosing samples as either normal or BV, but the intermediate flora presents problems [7,8]. Vaginal smears with intermediate flora may be considered as heterogeneous flora that most commonly include normal flora rather than BV, according to the definition of Amsel criteria [22]. Furthermore,

recent PCR assays report the heterogeneous character of intermediate flora, with some of them suggesting a molecular profile more similar to that of BV than to normal samples [13,14].

The molecular criteria's lower positive predictive value of 73% suggests that some positive molecular results are false positives (5.5%), i.e., although the molecular profiles of *G. vaginalis* and *A. vaginae* are identical to those seen in BV patients, the women do not meet the clinical (≥ 3 Amsel criteria) or microbiologic criteria (Nugent score ≥ 7) that define BV (Table 2). Alternatively, however, these results may represent true positives for the molecular condition of BV that were missed by traditional diagnostic tools. The false negatives of both standard methods reported above may support this alternative explanation. In any case, our molecular tool, based on PCR quantification of *G. vaginalis* and *A. vaginae*, clearly defines a reproducible and standardized molecularly defined BV, irrespective of the clinical and microscopic characteristics of vaginal flora. The clinical implications of this molecular condition should help to improve our understanding of BV, especially during pregnancy. Thus, we recently reported that high vaginal concentrations of *G. vaginalis* and *A. vaginae* are associated with a significant risk of preterm delivery in women with preterm labor [23].

Some points must be discussed as potential limitations of our study. First, we did no attempt to test for each bacterial species known to be found in the vagina, but the spectrum of targeted microorganisms is significant in BV. It is possible that quantification of additional vaginal microorganisms would modify the results. Second, repeat vaginal sampling to detect changes in the vaginal flora could provide valuable information about the pathogenesis and natural history of the molecular condition of BV. **Third, quantitative PCR assays have recently demonstrated a substantial variability in *G. vaginalis* levels with menstrual cycle [24].**

Consequently, the same threshold established in amenorrheic pregnant women may not be appropriate for non-pregnant women. In conclusion, comparison with the reference methods of the Amsel criteria and the Nugent score shows that the quantitative real-time PCR assay, targeting *G. vaginalis* and *A. vaginae,* is a reliable tool for the diagnosis of BV. The pathogenesis of the molecular condition of BV should be investigated.

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Disclosure of interests.

Université de la Méditerranée has applied for a patent on the quantitative molecular tool used in this study. European Patent Office N° 2087134.

Ethics approval

Institutional Review Board (Ethics committee of the University Aix-Marseille, France) approved the study (in a decision dated September 19, 2008).

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Table

Table 1: Characteristics of vaginal flora according to the Amsel criteria and Nugent score.

Methods for diagnosis	Women with bacterial vaginosis	Women without bacterial vaginosis	
		Normal flora	Intermediate flora
Amsel criteria	20 (12%)	143 (88%)	-
Nugent score	20 (12%)	117 (72%)	26 (16%)
Amsel criteria and Nugent score	15 (9.2%)	117 (72%)	-
Amsel criteria or Nugent score	25 (15%)	117 (72%)	21 (13%)

Table 2: Molecular characteristics of vaginal flora for women with bacterial vaginosis (n=25) and without bacterial vaginosis (n=138), with the Amsel criteria and Nugent score as reference methods.

PCR quantification (bacteria/mL)	Women with bacterial vaginosis n=25	Women without bacterial vaginosis n=138	
		Normal flora n=117	Intermediate flora n=21
No *Lactobacillus* species	18 (72%)	8 (6.8%)	6 (28%)
G. vaginalis $\geq 10^9$/mL	18 (72%)	0	4 (19%)
A. vaginae $\geq 10^8$/mL	24 (96%)	0	6 (28%)
G. vaginalis $\geq 10^9$/mL and/or *A. vaginae* $\geq 10^8$/mL	25 (100%)	0	9 (43%)

Table 3: Molecular loads for *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae* for women with bacterial vaginosis (n=25) according to Amsel criteria (≥ 3 criteria) or Nugent score (score ≥ 7).

	Method for diagnosis		PCR quantification (bacteria/mL)		
N	Amsel criteria	Nugent score	*Lactobacillus*	*G. vaginalis*	*A. vaginae*
1	**BV**	**BV**	-	1.15×10^{9}	1.09×10^{8}
2	**BV**	**BV**	1.83×10^{3}	2.69×10^{10}	5.31×10^{8}
3	**BV**	**BV**	-	1.19×10^{9}	8.73×10^{8}
4	**BV**	**BV**	-	4.47×10^{8}	8.34×10^{10}
5	**BV**	**BV**	-	4.73×10^{9}	4.84×10^{9}
6	**BV**	**BV**	2.38×10^{8}	-	1.40×10^{9}
7	**BV**	**BV**	-	6.32×10^{8}	2.30×10^{9}
8	**BV**	**BV**	-	1.88×10^{9}	7.20×10^{8}
9	**BV**	**BV**	9.20×10^{5}	3.10×10^{9}	1.07×10^{9}
10	**BV**	**BV**	-	1.80×10^{9}	2.20×10^{9}
11	**BV**	**BV**	-	2.60×10^{8}	7.60×10^{8}
12	**BV**	**BV**	-	7.75×10^{9}	1.05×10^{8}
13	**BV**	**BV**	-	1.40×10^{9}	1.30×10^{8}
14	**BV**	**BV**	-	3.40×10^{9}	1.50×10^{9}
15	**BV**	**BV**	5.67×10^{8}	3.05×10^{9}	6.16×10^{9}
16	**BV**	IF	2.38×10^{9}	-	2.04×10^{9}
17	**BV**	IF	-	1.02×10^{9}	4.80×10^{8}
18	**BV**	IF	-	5.67×10^{9}	1.52×10^{9}
19	**BV**	IF	-	3.42×10^{9}	6.87×10^{9}
20	**BV**	IF	-	1.80×10^{9}	1.80×10^{8}
21	NF	**BV**	-	5.30×10^{9}	1.14×10^{9}
22	NF	**BV**	-	-	2.04×10^{8}
23	NF	**BV**	-	1.56×10^{10}	1.05×10^{9}
24	NF	**BV**	3.78×10^{8}	5.41×10^{9}	3.82×10^{7}
25	NF	**BV**	2.44×10^{7}	1.50×10^{8}	2.00×10^{8}

BV: Bacterial Vaginosis; NF: Normal Flora; IF: Intermediate Flora.

Table 4: Sensitivity, specificity and predictive values with 95% confidence intervals of the PCR quantification for the prediction of bacterial vaginosis among women with bacterial vaginosis (n=25) and without bacterial vaginosis (n=138), with the Amsel criteria and Nugent score as reference methods.

Microorganism threshold quantification to predict bacterial vaginosis	% (95% confidence intervals)			
			Predictive value	
	Sensitivity	Specificity	Positive	Negative
G. vaginalis $\geq 10^9$/mL	0.72 (0.54-0.90)	0.97 (0.94-0.99)	0.82 (0.66-0.98)	0.95 (0.91-0.99)
A. vaginae $\geq 10^8$/mL	0.96 (0.88-1)	0.96 (0.92-0.99)	0.80 (0.66-0.94)	0.99 (0.98-1)
G. vaginalis $\geq 10^9$/mL and/or *A. vaginae* $\geq 10^8$/mL	1	0.93 (0.89-0.97)	0.73 (0.59-0.88)	1